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A stereological study of the ependyma of the mouse spinal cord. With a comparative note on the choroid plexus ependyma

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INTRODUCTION

In the last ten years a rapid development in stereological methods has occurred and a number of papers describing procedures for calculating particle size, volume, surface area and length have been published (see Gundersen, 1986 and Gundersen *et al.* 1988*a*, *b* for review). Assuming a valid sampling scheme, these methods give the real values irrespective of the shape of the particle in question. As part of a study on the ependyma of the mouse spinal cord (Bjugn *et al.* 1989*a b*, *c*), we wanted to apply these stereological methods in order to quantify certain aspects of the ependyma. A comparative morphometric study involving the choroid plexus ependyma was therefore made. The comparison served as a morphologic basis for a discussion of the function(s) of the spinal cord ependyma, which is largely unknown.

MATERIAL AND METHODS

Preparative technique

Six female albino mice (Bom: NMRI, 28·8–34·0 g) were used. All animals were anaesthetised with an intraperitoneal injection of Equithesin^{R} (1 ml/100 g body weight). They were then subjected to vascular perfusion fixation (40 ml/min for 10 minutes) and postfixation using a modified Tyrode's solution (Romeis, 1948), containing 50% of the usual quantity of sodium chloride, 0·06 M sucrose and 3% glutaraldehyde (pH 7·2, 37 °C, 560 mosmol) (see Bjugn *et al.* 1988*a* for details). The brains and spinal cords were isolated and rinsed in buffer. Parts of the brain were embedded in 7% agar, vibratome sectioned (100 μm) and the choroid plexus in the third ventricle identified. Spinal cord sections (see below), together with the choroid plexus sections, were osmicated for two hours in 1% osmium tetroxide in buffer and rinsed in distilled water. Dehydration was carried out in ethanol, stepwise from 70 to 100%. Infiltration and embedding in epoxy resin were made with propylene oxide as the intermediate agent. Light microscopical sections, 1 μm thick, were stained with toluidine blue. Ultrathin sections for transmission electron microscopy (TEM) were stained with uranyl acetate and lead citrate.

Stereological techniques

(A) *The whole spinal cord*

After isolation of the spinal cords, the spinal nerves and cauda equina were removed. Length and weight of the cords were recorded. The volume of each cord was measured by fluid displacement. Each cord was then divided in three parts (Fig. 1). The first part, designated C, extended from the end of the medulla oblongata to the

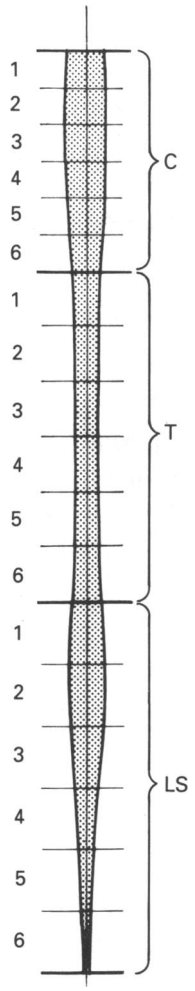

Fig. 1. Illustration showing how mice spinal cords were sectioned in order to obtain representative material from the cervical (C), thoracic (T) and lumbosacro-coccygeal (LS) parts of the cord.

end of the cervical enlargement. The second part, designated T, extended down to about halfway between the start of the lumbosacral enlargement and the widest part of this enlargement. The third part, designated LS, extended down to the end of the conus medullaris. The three segments correspond roughly, but not exactly, to the cervical, thoracic, and lumbosacro-coccygeal parts of the spinal cord. The length and weight of each part were measured. The parts were then transversely cut into six sections that were numbered consecutively (Fig. 1) and processed for microscopy.

(B) *Relative volumes of white matter, gray matter, ependyma and central canal in the spinal cord*

Survey micrographs of the spinal cord sections were taken at × 13 magnification according to the scheme given in Table 3. Corresponding micrographs of the ependyma and central canal were taken at × 130 magnification. The photographs were enlarged 6·5 times. The fraction of points falling on white matter, gray matter, ependyma and central canal were then measured by superimposing a lattice (16 × 19 cm) with 962 test points on the photographs (Weibel, 1980).

(C) *Relative volumes of intracellular organelles*

The volume fraction of nuclei, mitochondria, rough endoplasmic reticulum (RER), Golgi apparatus and lipid droplets in the ependyma, of both spinal cord and choroid plexus, were estimated by superimposing the above described lattice on electron micrographs taken at ×5600 magnification and enlarged 2·9 times. Electron micrographs representing the ependyma of the spinal cord were taken from the mid-cervical (section C3 or C4) and mid-lumbosacral enlargement (section LS2 or LS3). Care was taken to include entire cell heights in the photographic series. Only five of the six animals were included in this part of the study as the choroid plexus of one animal was destroyed during processing.

(D) *Mean cell volume of the ependyma in the spinal cord*

The mean ependymal cell volume was estimated by two different approaches:

(i) The total ependymal volume (see A, B) were divided on total number of ependymal cells as estimated by the disector principle (E(i) below).

(ii) The mean volume of ependymal nuclei was estimated on electron micrographs (from the mid-cervical and mid-lumbosacral enlargements (C)) enlarged ×2300 (×800 primary magnification) by 'point-sampling of linear intercept lengths' (Gundersen & Jensen, 1985; Brændgaard & Gundersen, 1986; Gundersen *et al.* 1988*b*). Given this volume, the mean cell volume could be estimated by knowing the cell volume fraction of the nuclei. This fraction was known from point sampling on electron micrographs (C).

(E) *Number of ependymal cells in the spinal cord*

The total number of ependymal cells in the spinal cord was estimated by two different approaches:

(i) The disector principle (Sterio, 1984): Two parallel semithin sections (1 μm) separated by 1 μm were sampled representing each section of the spinal cord (section C1, C2, T1, T2, LS1, LS2 from animals three and six, section C2, C5, T2, T5, LS2, LS5 from animals two and five, and section C3, C6, T3, T6, LS3, LS6 from animals one and four). In each section the ependymal area was photographed (B). All ependymal nuclei (each representing one cell) present in the first section but *not* in the second were counted. Likewise, all nuclei present in the second section but *not* in the first were counted. The mean value of these two numbers represents the number of ependymal cells present along 1 μm of the spinal cord. Knowing the length of the spinal cord (A), one could estimate the total number of ependymal cells in the spinal cord.

(ii) The selector principle (Cruz–Orive, 1987): The total volume of ependyma (A and B) was divided on mean volume of ependymal cells estimated by 'point-sampling of intercept lengths' (D(ii)).

Statistical methods

Statistical analysis was done using the Wilcoxon two-sample test or Kruskal–Wallis multiple sample test. Level of significance was taken to be $P < 0{\cdot}05$.

RESULTS

The spinal cords had a mean volume of 98 μl. The mean weight was 105 mg and the mean length 44 mm (Table 1). The lengths and weights of the three parts (C, T and LS), into which the spinal cords were divided, are given in Table 2. The sum of the

Table 1. *Volume, weight and length of mouse spinal cords devoid of spinal nerves*

Animal code	Weight (g)	Spinal cord		
		Volume (μl)*	Weight (mg)	Length (mm)
1	29·9	95	105	44
2	29·8	105	106	43
3	29·3	100	107	43
4	34·0	100	111	45
5	28·8	90	94	45
6	30·6	100	109	46
Mean	30·4	98	105	44

* Volumetric measurements are estimated to nearest 5 μl.

Table 2. *Weights (w) and lengths (l) of the three parts (C, T and LS) of the mouse spinal cords (compare Table 1 and Figure 1)*

Animal code	Part					
	C		T		LS	
	w (mg)	l (mm)	w (mg)	l (mm)	w (mg)	l (mm)
1	35	10	29	13	41	21
2	33	9	34	15	38	19
3	39	11	32	14	38	18
4	36	10	35	15	37	22
5	34	11	23	13	38	22
6	37	11	31	15	38	21
Mean	36	10·3	31	14·2	38	20·5

weights and lengths of the three parts never varied more than 3 mg and 2 mm respectively from the values recorded on the corresponding intact cord.

Using the disector principle (E(i)), the number of ependymal cells along the spinal cord was found to be 3·5/μm length in the C part, 3·1/μm in the T part and 4·2/μm in the LS part of the cord. Combining these values with the mean length of the three parts (Table 2), the total number of cells was found to be 166000 (e.g. for the C part; 3·5 cells/μm × 10·3 mm = 36000 cells). (When taking shrinkage during processing into account, the number of ependymal cells should be corrected to 150000; see Discussion.) Using the selector principle (E(ii)), the total number of ependymal cells was calculated to be 176000.

Estimating the total spinal cord ependymal volume to be 83×10^6 μm³ (see above) and the total number of ependymal cells to be 166000 as calculated by the disector principle (see above), the mean ependymal cell volume was found to be 500 μm³ (550 μm³ when assuming 150000 cells, see above and Discussion).

Using 'point-sampling of intercept lengths' for estimating the mean nuclear volume of ependymal cells (D(ii)), a value of 92 and 104 μm³ was found for the cervical and lumbosacral nuclei respectively. Combining these values with the calculated nuclear fraction of ependymal cell volume (Table 4), the mean ependymal cell volume was estimated to be 350 μm³. (When taking shrinkage during processing into account, this value will be approximately 470 μm³; see Discussion.)

The relative volumes of the white matter, gray matter (including ependyma and

Table 3. *Relative volumes of white and gray matter (including ependyma and central canal), ependyma and central canal at various levels of the mouse spinal cord as estimated by point-counting*

Animal code	Level	Points per section	White matter (%)	Gray matter (%)	Ependyma (‰)	Central canal (‰)
3	C1	81400	52·7	47·3	0·70	0·10
6	C1	85800	51·3	48·7	0·59	0·09
2	C2	90200	61·0	39·0	0·53	0·16
5	C2	85800	61·5	38·5	0·57	0·10
1	C3	86900	53·2	46·8	0·49	0·09
4	C3	82500	57·3	42·7	0·72	0·32
3	C4	84280	61·2	38·8	0·35	0·11
6	C4	86900	58·2	41·8	0·36	0·05
2	C5	75680	53·4	46·6	0·37	0·09
5	C5	88000	51·3	48·7	0·55	0·18
1	C6	64500	57·3	42·7	0·45	0·11
4	C6	59340	60·1	39·9	0·94	0·46
	Mean		56·5	43·5	0·55	0·16
3	T1	44290	61·2	38·8	0·81	0·09
6	T1	58050	70·4	29·6	0·52	0·05
2	T2	43860	62·7	37·3	0·41	0·16
5	T2	43430	68·3	31·7	1·11	0·28
1	T3	45580	62·3	37·7	0·53	0·09
4	T3	41280	69·8	30·2	1·26	0·44
3	T4	38270	70·8	29·2	0·63	0·05
6	T4	44290	65·0	35·0	0·68	0·16
2	T5*	—	—	—	—	—
5	T5	40420	66·0	34·0	0·84	0·15
1	T6	49020	64·9	35·1	0·43	0·06
4	T6	48160	58·0	42·0	1·20	0·42
	Mean		65·4	34·6	0·69	0·18
3	LS1	52460	57·4	42·6	0·80	0·15
6	LS1	55470	55·8	44·2	0·83	0·11
2	LS2	66220	52·6	47·4	0·80	0·12
5	LS2	60200	56·4	43·6	0·85	0·23
1	LS3	73960	47·7	52·3	0·42	0·08
4	LS3	45580	47·2	52·8	1·12	0·22
3	LS4*	—	—	—	—	—
6	LS4	38270	55·1	44·9	1·38	0·31
2	LS5	22360	38·5	61·5	2·37	0·72
5	LS5	23220	38·9	61·1	2·33	0·47
1	LS6	36960	34·9	65·1	0·49	0·14
4	LS6	13760	34·4	65·6	2·62	1·60
	Mean		47·2	52·8	1·27	0·38

* Section destroyed during preparation.

central canal), ependyma and central canal at different levels of the spinal cord are given in Table 3. The ependyma made up 0·55, 0·69 and 1·27 ‰ in the C, T and LS part respectively. Using the mean value (0·84 ‰), the total ependymal volume in the spinal cord was estimated to be 82×10^6 μm^3 (0·84 ‰ × 98 μl). Considering each part of the cord separately, the total ependymal volume was estimated to 84×10^6 μm^3 (e.g. for the C part; 0·55 ‰ × 0·036 g × 933 μl/g = $18{\cdot}5 \times 10^6$ μm^3. The mean weight of the cervical part was taken from Table 2. The mean volume/weight ratio was calculated from Table 1).

Applying similar approaches to estimate the volume of the central canal, a value of 24×10^6 μm^3 was calculated by both methods.

Table 4. *The relative volume fraction of certain intracellular constituents of the ependyma at the mid-cervical and mid-lumbosacral enlargements of the mouse spinal cord and of the choroid plexus of the third ventricle as estimated by point-counting. Values are given as %*

Animal code	Points counted	Nuclei	Mitochondria	RER*	Golgi apparatus	Lipid droplets
			Mid-cervical level			
1	3482	29·0	5·6	0·3	1·1	0·4
2	2967	35·8	4·7	0·4	0·8	0·9
3	2471	24·3	5·6	0·2	0·7	0·6
4	4298	25·4	4·6	0·5	1·0	0·6
5	2869	19·8	4·7	0·4	0·9	0·5
Mean		26·9	5·0	0·4	0·9	0·6
			Mid-lumbosacral level			
1	4121	28·1	5·0	0·6	0·7	0·1
2	3631	30·0	4·4	0·2	0·4	0·3
3	3745	28·0	4·4	0·1	0·8	0·6
4	4449	35·1	3·0	0·5	0·8	0·4
5	4952	26·0	4·1	0·4	1·0	0·5
Mean		29·4	4·2	0·4	0·7	0·4
			Choroid plexus			
1	3707	15·7	8·3	3·4	0·3	0
2	5257	8·8	10·8	1·5	0·5	0
3	4452	20·7	9·9	2·0	0·4	0
4	4266	13·5	10·0	2·0	0·5	0
5	3941	13·7	10·1	1·6	0·2	0
Mean		14·5	9·8	2·1	0·4	0

*RER, rough endoplasmic reticulum.

The relative volumes of certain intracellular constituents in the ependyma, both of spinal cord and choroid plexus, are given in Table 4. Applying the Wilcoxon two-sample test, no statistically significant volume fraction differences regarding the constituents were found between the ependyma at the cervical and lumbosacral level of the spinal cord (Table 4). The relative volume fractions of mitochondria and rough endoplasmic reticulum (RER) in the choroid plexus ependyma were found to be significantly larger ($P < 0{\cdot}01$) than in the spinal cord when applying the Kruskal–Wallis multiple sample test. Likewise, the relative volume fractions of nuclei and Golgi apparatus were found to be significantly larger in the spinal cord ependyma than in the choroid plexus ($P < 0{\cdot}025$ and $P < 0{\cdot}01$ respectively).

Although we do not wish to describe the ultrastructural details in this report, it is worth mentioning that the choroid plexus mitochondria, in addition to constituting a larger volume fraction of the ependyma, generally appeared larger and had more numerous and elaborate cristae than their counterparts in the spinal cord ependyma.

DISCUSSION

Stereological aspects

Even though the applied methods are unbiased, the preparative techniques (fixation, dehydration, embedding, sectioning) and the measurements (volumes, lengths, numbers) will cause our estimates to deviate in some degree from the 'true'

(i.e. in the living animal) values. Some of these possible errors will be discussed in further detail.

In the recordings on the spinal cord, the volume displacement was the most difficult one. The uncertainty was $\pm 2{\cdot}5\ \mu l$. This will cause less than a 3% variation in the estimation of the total ependymal volume. Some uncertainty was involved in deciding the border between ependyma and surrounding neuropil on the light micrographs. The uncertainty involved in decision-making on one particular micrograph would be negligible for the estimation of total ependymal volume as the number of observations will cancel out single errors.

According to Boyde & Boyde (1980), a stepwise ethanol dehydration will cause osmicated adult mouse brain blocks to shrink to 75% of the volume found after the initial glutaraldehyde fixation (implicating a linear shrinkage of approximately 10%). Taking this shrinkage into consideration, the number of ependymal cells estimated by the disector principle (166000) should be corrected to 150000 as the measurements were made in dehydrated and resin-embedded tissue. Correspondingly the mean ependymal cell volume should be corrected to 550 μm^3. Using 'point-sampling of linear intercept lengths' (D(ii)), a mean ependymal cell volume of 350 μm^3 was estimated. This technique was also applied on dehydrated and plastic-embedded specimens. The volume of 350 μm^3 should therefore be corrected to 470 μm^3.

To our knowledge, only one study concerning the volume and number of ependymal cells in the mouse spinal cord has been published. In adult albino mice the total ependymal volume in the cervical part of the spinal cord was found to be $6{\cdot}9 \times 10^6$ μm^3 and the number of cells 20600 (Sakla, 1965). In the present study, the ependymal volume in the C part was calculated to be $18{\cdot}5 \times 10^6$ μm^3 and the number of cells to be 36000 (32000 when taking shrinkage into consideration, see above). We believe that the differences found are due to the different methodological approaches.

Functional aspects

The cell volume fraction of mitochondria was 2·1 times larger in the choroid plexus ependyma (9·8%) than in the spinal cord ependyma (4·6%). As the choroid plexus mitochondrial cristae were also more elaborate, an indication of high metabolic activity (Ernster & Schatz, 1981), one may assume a significantly higher oxidative metabolic activity in the choroid plexus ependyma than in the ependyma of the spinal cord. Tissue with high oxidative metabolic activity, such as liver and heart muscle, contains a five to ten times larger mitochondrial volume fraction than that found in the spinal cord ependyma (see David, 1977). The relative cell volume fraction of RER was more than five times larger in the choroid plexus (2·1%) than in the spinal cord ependyma (0·4%). RER is usually abundant in cells specialised for protein secretion, such as pancreatic acinar cells and liver cells. In these cells, the RER volume fraction is however at least 20 times larger than that found in the spinal cord ependyma (see David, 1977).

SUMMARY

Applying different stereological techniques, the total ependymal volume in the spinal cord of mice was estimated to be 83×10^6 μm^3, the number of cells to be 163000 and the mean ependymal cell volume to be 510 μm^3. Compared to choroid plexus cells in the third ventricle, the ependymal cells in the spinal cord contained a smaller mitochondrial volume (9·8% versus 4·6% of cell volume) and less rough endoplasmic

reticulum (2·1 % versus 0·4 %). These findings indicate that the metabolic activity of the ependyma in the spinal cord is lower than that in the choroid plexus. Compared to liver and exocrine pancreatic cells, ependymal cells in both locations must be considered to have a rather low metabolic activity.

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